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Molecular phylogenetic study in genus *Hydra*

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Abstract

Among 8000 - 9000 species of Cnidaria, only several dozens species of Hydrozoa have been found in the fresh water. Hydra is such a fresh water polyp and has been used as a good material for research in developmental biology, regeneration and pattern formation. Although the genus *Hydra* has only a few ten species, its distribution is cosmopolitan. The phylogenetic relationship between hydra species is fascinating from the aspect of evolutionary biology and biogeography. However, only a few molecular phylogenetic studies have been reported on hydra. Therefore, we conducted a molecular phylogenetic study of the genus *Hydra* based on mitochondrial and nuclear nucleotide sequences using a hydra collection that has been kept in the National Institute of Genetics (NIG) of Japan. The results support the idea that four species groups comprise the genus *Hydra*. Within the *viridissima* group (green hydra) and *braueri* group, genetic distances between strains were relatively large. In contrast, genetic distances between strains among the *vulgaris* and *oligactis* groups were small irrespective of their geographic distribution. The *vulgaris* group strains were classified at least (as far as our investigated samples) into three sub-groups, *vulgaris* sub-group, *carnea* sub-group, and *H. sp.* (K5&K6) sub-group. All of the *vulgaris* sub-group and *H. sp.* (K5&K6) sub-group strains were collected in Eurasia. The *carnea* sub-group strains in NIG collection were all collected in North America. A few newly collected samples in Japan, however, suggested belonging to the *carnea* sub-group according to the molecular phylogenic analysis. This suggests a trans-Pacific distribution of the *carnea* sub-group hydra.

Key Words: genus *Hydra*, molecular phylogeny, mitochondrial DNA, nuclear DNA

1. Introduction

Hydra, a member of phylum Cnidaria, class Hydrozoa, order Hydroida, family Hydridae, is a fresh water polyp that has no medusa stage and usually reproduces by budding asexually. Because of its simple body plan and remarkable regeneration activity, hydra has been used as one of the best model animal in developmental biology, especially in study on pattern formation (Bosch and Fujisawa, 2001; Bosch and Khalturin, 2002; Steele, 2002; Holstein et al, 2003) and on the stem cell system (Bosch, 2009; Bosch et al., 2010).

Most of eumetazoans have bilateral symmetric body that consist of three germ layers and are referred as Bilateria. In the other eumetazoans, the Cnidaria is the largest and most diverse group. Its body wall consists of only two germ layers, ectoderm and endoderm. In Cnidaria, Anthozoa species have bilateral symmetry in the internal morphology whereas Medusozoa (Scyphozoa, Cubozoa, and Hydrozoa) species have radial or tetra radial symmetry body plan (Nielsen, 2001). Therefore cnidarian has been attracting interest from researchers in animal phylogeny and evolutionary biology, especially from the aspect of origin of the body plans in animals.

The Cnidaria comprise 8,000 – 9000 living species (Tudge, 2000), which occur mostly in marine aquatic habitats. Only several dozens species occur in fresh water habitats. (Dumont 1994, Jankowski, Collins and Campbell, 2008). Most of the fresh water cnidarian belong to the Hydrozoa, fresh water medusae (genus *Limnocnida* and *Craspedacusta*) and solitary polyp, hydra (genus *Hydra*). It is not unclear whether all freshwater cnidarians have a same origin or not. In the reported cnidarian molecular phylogenetic studies, however, species of hydra always formed a monophyletic group (Bridge et al, 1995; Collins, 2000 & 2002; Collins et al., 2006). These suggest that an ancestor of hydra had made its way into fresh water and later that ancestor speciated into additional species. Although only a few phylogenetic studies on species in the genus *Hydra* have been reported, Campbell (1983, ’87 and ’89) proposed that genus *Hydra* consists of four species groups, *viridissima* group, *braueri* group, *vulgaris* group, and *oligactis* group, based mainly on the morphological characters. Jankowski *et al*. (2008) suggested that the *viridissima* group and the *vulgaris* group were probably present before the continents separated. They also suggested that the *oligactis* group and the *braueri* groups are restricted to the northern continents and presumably arose after the separation of northern and southern landmasses. In this manner, the origin of fresh

water cnidarians and their diversity across all continents pose intriguing problems. To solve these problems, molecular phylogenetic studies of freshwater cnidarians are indispensable.

Only a few molecular phylogenetic studies, however, have been reported. Thomsen and Bosch (2006) reported differences in gene number and structure within the PPOD (foot-specific peroxidase) gene family in hydra species. Their results supported Campbell's idea of four groups and also suggested unexpected genomic variation within closely related species of hydra. Hemmrich et al. (2007) estimated the phylogenetic relations with molecular data from two nuclear (18S rDNA, 28S rDNA) and two mitochondrial (16S rRNA, cytochrome oxidase subunit I (COI)) genes among eight members of the genus *Hydra*, which have been kept in laboratories and often used for many researches. Their results also supported Campbell's idea; genus *Hydra* consists of four species groups, *viridissima* group, *braueri* group, *vulgaris* group, and *oligactis* group. Using molecular phylogenetic analyses, they found that in contrast to its initial description, the strain, *Hydra vulgaris* (strain AEP), used for making transgenic hydra (Wittlieb *et al.*, 2006), is more closely related to *H. carnea* than to the other species of hydra and stated the difficulty of identifying hydra species based only on the morphological characters. Therefore, molecular phylogenetic studies on the genus *Hydra* are eagerly anticipated, as they will provide basic data for diverse studies on hydra.

Mitochondrial DNA (MtDNA) has been used extensively for the molecular phylogenetic analysis in a wide range of organisms. MtDNA in Medusozoa (Schiphozoa, Cubozoa, Hydrozoa) displays in the form of a linear molecule (Bridge, Schierwater, DeSalle, Buss, 1992) while most of organisms, including the Anthozoa, have circular form MtDNA. In two species of hydra, *H. oligactis and H. maganipapillata*, mitochondrial DNA sequences have been determined recently (Kayal and Lavrov, 2008; Voigt, Erpenbeck and Wörheide, 2008). They showed that *H. oligactis* has one linear MtDNA and *H. maganipapillata* has two fragments of linear MtDNA. Therefore, analyzing MtDNA in hydra species is intriguing with the both object of phylogeny of hydra and evolution of mitochondrial DNA itself.

National Institute of Genetics Japan maintains a hydra strain collection that includes several dozens strains (identified as several species). Using this collection and some newly collected hydra specimens in Japan, we studied molecular phylogenies in

the genus *Hydra* with molecular data from mitochondrial (*lrRNA, tRNA, cytochrome oxidase subunit II*; *COII*, and *cytochrome oxidase subunit I*; *COI*) DNAs, and two nuclear genes (*CnNOS1*, *EF1a*).

2. Materials and methods

2.1. Hydra strains and out groups for analysis

Molecular phylogenetic analyses were carried out with several ten hydra strains that have been kept in the National Institute of Genetics (NIG) of Japan and some specimens collected in Fukuoka prefecture in Japan.

All *H. magnipapillata* strains (A1;nem-3, A9;sf-1, B4, B8;105, B10;identified as *H. japonica*, B11, B12, C2, D1;mini-1, D5;nem-11, D7;maxi-1, E4, E6, F2;reg16, J1, J2, J6, J7, J10) had been collected in Japan. *H. vulgaris* K9 strain originated in Europe and AEP, and M5 strains originated in USA. *Hydra attenuata* (B6, K5, K6, K7, L2) originated in Europe and *H. carnea* (L4) in USA. *Hydra oligactis* G7 strain originated in Germany and M2, M4 strains in USA. *Hydra fusca* (K12) was of European origin. *Pelmatohydra robusta* (Japanese *oligactis* group hydra, B1, B2, B3, L5) had been collected in Japan. *Hydra circumcincta* strains (K8, M7) originated in Europe and *H. utahensis* (L10) and *H. hymanae* (M1) in USA. Some of *H. viridissima & viridis* strains (B5, K10, M9, M10, N11) originated in Europe and L9, M8 strains in USA. Sampling location of *Hydra sp.* (J8) has been unknown. All these strains have been kept at NIG and were used for the molecular phylogenetic analyses in this study. Species identification of the underlined strains, K5, K6, M2, and M5, were suggested to be inaccurate as discussed later in this report. Therefore, we referred these strains as *Hydra sp.* in some cases.

The polyps were cultured under the standard condition. Each strain was kept in a plastic dish filled with a hydra culture solution (1 mM NaCl, 1 mM CaCl2, 0.1 mM KCl, 0.1 mM MgS04, 1 mM tris-(hydroxymethyl)-aminomethane; pH 7.4, adjusted with HCl) at 20ºC. The polyps were fed with newly hatched nauplii of Artemia two times a week.

We collected polyps in 5 ponds at 4 locations, , Tanushimaru, Kasuga, Tajima, and Ohori (FT 08, KAS 20, ONI 5, FO1 G, FO2 03, FO2 05), in Fukuoka prefecture in Japan. All polyps collected were identified as belonging to the *vulgaris* group species

based on morphological traits by the stereoscopic microscopy observation. These several dozens polyps were also used for the molecular phylogenetic analyses. FT 08 represented 4 samples that had been collected at the same location and showed the same nucleotide sequence. Similarly, KAS 20, ONI 5, FO1 G, FO2 03, and FO2 05 represented 12, 6, 12, 3, and 3 samples respectively.

The NCBI genomic data of marine hydrozoans Anthozoa *Nematostella vectensis* (DQ643835, AY730693, AB126336) served as out groups in the phylogenetic analyses.

2.2. DNA isolation, PCR amplification and sequencing

Genomic DNA and mitochondrial DNA from each strain was isolated using a genomic DNA extraction Kit (DNeasy Tissue Kit, QIAGEN)). To obtain a DNA template for the PCR amplification from one polyp, a polyp was homogenized in a 50 µl PCR buffer (50mM KCl, 10mM tris-hydroxymethyl-aminomethane HCl pH8.3, 0.1% NP-40), treated with proteinase K (add 2µl 20mg/ml proteinase K stock solution, Takara Bio Inc.) at 55^{o}C for one hour and then incubated at 95^{o}C for 15 minutes to inactivate the proteinase K. The target DNA fragments (partial sequences of mitochondrial genes and some nuclear genes) were PCR amplified using degenerate primers and heterologous primers designed for each genes based on the MtDNA sequence of *H. magnipapillata* (GenBank Accession #AF100773.1), CnNOS1 sequence of *H. magnipapillata* (GenBank Accession #AB037080.1), and EF1a sequence of *H. magnipapillata* (GenBank Accession #D79977.1). Primer sequences were as follows. For MtDNA *lrRNA-tRNA(W)-COII* locus, MtF 5'-GTGAAAAACTAATCGAGTTAAGAGA -3' and MtR 5'-CCACATAATTCTGAACATTGACC -3' were used. For MtDNA *COI*, G-COIF 5'-TGGTGCATTTTCTGGAATGATAGGTAC -3', G-COIR 5'-CTATCAGTTAGTAGCATAGTTATAGC -3' (for viridissima group), B-COIF 5'-CTGGCATTACTAAAAAAAATAT -3', and B-COIR 5'-AATATCATTACAAAAGCATGAGC -3' (for other groups) were used. For nuclear DNA, Nos1F 5'- AGTCAATTTTCTGTATTTCGTGA -3', Nos1R 5'-TGTTTTCGGTTTTGGAGTAT -3', EF1aF 5'- ATTTACAAATGTGGTGGTATCG -3', and EF1aR 5'- CCAATTTTGTAGACATCTTGAAG -3' were used.

The PCR amplified template DNA fragments were purified with Wizard SV Gel and PCR Clean-Up System (Promega). Sequence reactions were performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Samples were sequenced with ABI3100 Avant Genetic Analyzer (Applied Biosystems). All sequences have been submitted to DDBJ (Accession Nos. *CnNOS1*: AB565041-AB565065, *EF1a*: AB565066-AB565088, *COI*: AB565089- AB565143, *lrRNA-tryptophan (W) tRNA-COII*: AB565144 - AB565197).

2.3. Phylogenetic Analysis

Sequence alignments were generated using ClustalX (Thompson et al., 1997) or ClustalW (Thompson et al., 1994) included in MEGA4 soft ware package (Tamura et al., 2007; Kumar et al., 2008) or SeaView4.2 (Galtier et al., 1996). Alignments were optimized by hand using SeaView4.2 or MEGA4. Large insertions observed in MtDNA of L10 and M1 strains were deleted from the alignment. We used neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) methods to infer phylogenetic relationships among the hydra species and strains.

For the NJ method, evolutionary distances were computed using the Nei-Gojobori method (Nei and Gojobori, 1986) and are in the units of the number of synonymous differences per synonymous site for nuclear DNA (*CnNOS1, EF1a* genes). All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. There were a total of 188 positions in the final dataset of *CnNOS1* and of 204 positions in *EF1a*. For mitochondrial DNA the p-distances were used because these sequences included more than a half-length non amino acid sequences coding region, lrRNA and tRNA(W). All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. There were a total of 2439 positions in the final dataset. The evolutionary history was inferred using the NJ method (Saitou and Mei, 1987). Bootstrap consensus trees inferred from 10000 replicates were taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). We used MEGA4 for these analyses including the tree drawing.

We also used the MEGA4 for the maximum parsimony analyses. The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). All alignment gaps were treated as missing data.

There were a total of 574 positions in the final dataset, out of which 161 were parsimony informative for *CnNOS1* and a total of 612 positions in the final dataset, out of which 116 were parsimony informative for *EF1a*. For mitochondrial DNA, there were a total of 2439 positions in the final dataset, out of which 745 were parsimony informative. In supplemental Figures, most parsimonious trees are shown with the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates).

PhyML (Guindon and Gascuel, 2003) was executed using the GTR (General Time Reversible) model through SeaView4.2 for the maximum likelihood analyses. For selecting models we used jModelTest (Posada, 2008). Starting trees were made by BioNJ. For tree searching operation 'best of NNI (nearest-neighbor interchange) and SPR (subtree pruning and regrafting)' was applied. Bootstrap values were calculated (1000 replicates).

Using Mr. Bayes v. 3.12 (Huelsenbeck and Ronquist, 2001), we executed Bayesian inference analyses. GTR model and sets across-site rate variation for gamma distribution with a proportion of invariant sites were applied. We ran the analysis for 500,000 - 1000,000 generations and recorded the current tree and parameter values every 100 generations.

MEGA4 was also used to estimate synonymous substitution rates in the protein coding regions of mitochondrial DNA (*COI, COII* genes) and nuclear DNA (*CnNOS1, EF1a* genes). We used the Nei-Gojobori method (1986) for the estimation.

3. Results

3.1 Molecular phylogenetic analyses between species in genus *Hydra* using mitochondrial genes

Figure 1 shows a phylogenetic tree drawn based on the ML analysis on the combined data sets of mitochondrial genes, *COI* and *lrRNA-tRNA(W)-COII*. Partial sequences of *COI* gene (459 nucleotides) and partial sequences of *COII* gene (606 nucleotides) were in the amino acid sequence coding regions. This tree was drawn with corresponding sequences of *Nematostella vectensis* as an out-group.

Strains of *H. viridissima* and *H viridis* (B5, K10, L9, M8, M9, M10, N11) and *Hydra sp.* (J8), which possessed symbiont green algae in their endodermal epithelial cells, formed a clade and it was sister to all other strains. In both phylogenetic trees drawn by NJ and MP analyses (see Suppl. Fig.1A, B), these *viridissima* group strains also formed a monophyletic group sister to all other strains of hydra species. Only the result of BI analysis suggested that *braueri* group and *viridissima* group made a monophyletic clade (Supple. Fig. 1C). However, the posterior probability of this clade was not so high, 0.56.

All other strains diverged into three clades by the ML analysis (Fig. 1) and also by the NJ, MP, BI analyses (see Supple. Fig.1A, B, C). One of the clades consisted of the *braueri* group species (*H. circumcincta*; K8, M7, *H. utahensis*; L10, *H. hymanae*; M1). Another two clades were formed from the *oligactis* group (*H. oligactis*; G7, M4, *H. fusca*; K12, *Pelmatohydra robusta*; B1, B2, B3, L5, P) and the *vulgaris* group (*H. magnipapillata*; A1, A9, B4, B8, B10, B11, B12, C2, D1, D5, D7, E4, E6, F2, J1, J2, J6, J7, J10, *H. vulgaris*; K9, AEP, M5, *H. attenuata*; B6, K5, K6, K7, L2, *H. carnea*; L4, *H. sp.*; M2 and newly collected samples in Fukuoka, Japan; FO2 05, ONI 5, FO1 G, KAS20, FT 08, FO2 03), respectively. In the ML,NJ, MP, and BI analyses, the *oligactis* group strains and the *vulgaris* group strains formed a monophyletic clade (Fig.1 and Supple. Fig.1A, B, C) sister to the *braueri* group.

Figure 1 shows another distinct feature that the *vulgaris* group diverged into three sub-clades with very high bootstrap probabilities. One of them consisted of K5 and K6 strains that had been identified as *H. attenuata* in the NIG collection. Another clade consisted of L4 (had been identified as *H. carnea*), M5, AEP (had been identified as *H. vulgaris*), M2 (had been identified as *H. oligactis*) and newly collected samples (KAS 20, FT 08, FO2 03). All other strains of *vulgaris* group (*H. magnipapillata*; A1, A9, B4, B8, B10, B11, B12, C2, D1, D5, D7, E4, E6, F2, J1, J2, J6, J7, J10, *H. vulgaris*; K9, *H. attenuata*; B6, K7, L2, *H. sp.*; M2 and newly collected samples; ONI 5, FO1 G, FO2 05) formed still another clade (Fig.1). These three sub-clades were also recognized in NJ, MP, and BI trees (see Supple. Fig.1A, B, C). We named expediently these three sub-groups in *vulgaris* group, *H. sp (K5, K6)* sub-group, *carnea* sub-group, and *vulgaris* sub-group, respectively.

These molecular phylogenetic analyses also suggested that genetic distances between strains within the *vulgaris* group or within each sub-group were very small

irrespective of the geographic distribution of the used samples, Japan, Europe and North America. However, genetic distances between strains within the *braueri* group and *viridissima* group were not so small unlike the case of the *vulgaris* group.

3.2 Molecular phylogenetic analyses using nuclear DNAs, *CnNOS1* and *EF1a*, confirmed the inferred phylogenetic relationships in the genus *Hydra* based on the mitochondrial genes

We reconfirmed that hydra species clustered into the four monophyletic groups, the *viridissima* group, *braueri* group, *oligactis* group and *vulgaris* group based on the nuclear DNA sequence data analyzed by ML, NJ, and MP methods (Fig. 2, 3 and Supple. Fig. 2A, 2B, 3A, 3B). Only the result of BI analysis suggested *viridissima* group branched off into two clades (Supple. Fig. 2C, 3C). However, monophyly of the other three groups against *viridissima* group were also suggested by BI analysis as is the cases with ML, NJ, and MP analyses.

We also reaffirmed that the *vulgaris* group diverged into the three sub-groups, the *vulgaris* sub-group, *carnea* sub-group, and *H. sp. (K5, K6)* sub-groups (Fig. 2, 3 and Supple. Fig. 2A - C, 3A - C).

3.3 Occurrence of large insertions of mitochondrial DNA has been presumed in hydra species among *braueri* group

In the course of sequencing the mitochondrial DNA, we found large insertions in the *braueri* group hydra species. In *Hydra utahensis* (L10), a 1297 bp insertion was found between the *lrRNA* coding region and the *tRNA(W)* - *COII* coding region. 72 bp sequence in anterior part of this insertion showed 82% identity with the *tRNA(W)* region. Following it, from the 77th nucleotide sequence position a 1200bp open reading frame (ORF) was recognized. Anterior part of 47 amino acid sequence of predicted translated product of this ORF showed 57% identity and 68% similarity with the COII amino acid sequence. Many fragments in the following part also showed similarity with parts of the COII (Supple. Fig. 4).

In *Hydra hymanae* (M1), behind the 9th nucleotide of the *COII* coding region, a 500 bp insertion was observed. However, this insertion did not have any large ORF and we could not fined any homology or similarity with known sequences.

3.4 Morphological aspects of K5 and K6 strains

In *vulgaris* group, both the *vulgaris* sub-group and the *carnea* sub-group consisted of well known strains, such as a standard wild type strain *Hydra magnipapillata* 105 (B8) whose whole genomic sequence has been determined, strain AEP which was one of the most important strain for the future research because of its usefulness in transgenic hydra production. Polyps between these two sub-groups have shared common morphological traits, tentacles' length being longer than the body length, characteristic size and shape of nematocysts, holotrichous isorhizas with narrowly oval shape and transverse coils in the upper half.

K5 and K6 strains have been identified as *Hydra attenuata* along with B6, K7, and L2 strains in the NIG stock list. The B6, K7, L2 strains belonged the *vulgaris* sub-group in the present molecular phylogenetic analyses (Fig. 1 and Supple. Fig.1A, B, C), and their morphological traits resemble those of the *vulgaris* and *carnea* sub-group strains. K5 and K6 strains, however, have similar or shorter tentacles' length compared with the body length (Fig. 4A). The characteristics of holotrichous isorhizas with narrowly oval shape and transverse coils in the upper half are common with the other *vulgaris* sub-groups. However, the width of the holotrichous isorhiza was nearly half of the length, and the horizontal coils have only a few turns. Relatively larger size of desmoneme was another specific character. Its length was longer than atrichous isorhiza and similar to that of holotrichous isorhiza (Fig. 5). These morphological traits of K5 and K6 strains support independently the sub-group clustering of these strains in the *vulgaris* group along with the results of molecular phylogenetic analyses (Fig. 1 – 3 and Supple. Fig. 1 - 3).

3.5 Substitution rate difference between mitochondrial DNA and nuclear DNA in hydra

Using the *COI* and *COII* nucleotide sequence as representatives of mitochondrial genes, we estimated synonymous substitution rates in some of the hydra strains. For the estimation, we used the modified Nei-Gojobori method with the Jukes-Cantor correction. Partial nucleotide sequences of *CnNOS1* and *EF1a* were used as nuclear gene representatives. We compared the synonymous substitution rate between the MtDNA (*COI, COII*) and nuclear DNA (*CnNOS1, EF1a*) among several strains of hydra (Table 1). Both substitutions in the mitochondrial genes and nuclear genes between groups of hydra (*viridissima*, *braueri*, *oligactis* and *vulgaris* groups)

were considered already saturated. In contrast, between closely related strains such as between M9 – M10 and K8 – M7, almost no substitutions occurred,. Therefore, we compared the substitution rates between strains belonging to the same group but not so closely related to each other, such as L4 and L2 in the *vulgaris* group, and G7 and B3 in the *oligactis* group. Our results suggested that the substitution rate in the mitochondrial genome was 2.4-7.8 times higher than that of the nuclear genome in hydra (Table 1).

4. Discussion

4.1 Genus *Hydra* consist of four distinct species groups

Campbell (1987) suggested that hydras fit naturally into four clusters of species termed, the *oligactis*, *vulgaris*, *viridissima*, and *braueri* groups, based on several phenotypic characters. According to Campbell's keys (1983, 1987), species of the *viridissima* group are characterized by intracellular algal symbiont in the endodermal epithelium and small stenotele nematocysts not longer than 11 μm (Fig. 5). Taxonomic keys to the *braueri* group members are plump (at least half as broad as long) holotrichous isorhiza (Fig. 5) and embryo theca without spines. The *oligactis* group is characterized by their buds that acquire 2 lateral tentacles before other tentacles appear; otherwise, stenotele nematocysts at least 1.5 times as long as its width. Holotrichous isorhiza without transverse coils (Fig. 5) is also a trait of the *oligactis* group species except for *Hydra pseudoligactis* (=*Hydra canadensis*). The *vulgaris* group is characterized by its buds acquiring tentacles almost synchronously; otherwise, stenotele nematocysts less than 1.5 times as long as its width. Holotrichous isorhiza with transverse coils and slender shape is also a trait of the *vulgaris* group.

All strains of hydra species that were used in this study could be categorized into the four groups by Campbell's keys. Both molecular phylogenetic analyses using the mitochondrial genes *COI*, *lrRNA-tRNA(W)-COII* and nuclear genes *CnNOS1*, *EF1a* indicated that all analyzed hydra species were clustered into four monophyletic groups (Fig. 1 - 3 and Supple. Fig. 1 - 3). This clustering of four monophyletic species groups is consistent with Campbell's suggested grouping, the *viridissima* group, *braueri* group, *oligactis* group and *vulgaris* group. Although only the M2 strain had been identified as an *oligactis* group species (labeled *H. oligactis* on the list of the NIG collection), our molecular phylogenetic analyses suggested that it was a member of the *carnea*

sub-group in the *vulgaris* group. Then, we observed morphological key characters (structure of holotrichous isorhiza) of M2 strain polyps, and confirmed that M2 is a member of the *vulgaris* group (Fig. 5). Therefore, the presence of four monophyletic distinct species groups in the genus *Hydra* was affirmed based on the both morphological data and nucleotide sequence data. The molecular analysis on the eight members of the genus *Hydra* by Hemmrich et al. (2007) also supports the presence of four distinct species groups in hydra.

However, more than half of the strains used in this study originally had been collected in Japan. The sampling locations of the other strains were restricted to Europe and North America. Although, the genus *Hydra* has only a few dozens species, they have been recorded from all over the world excepting Antarctica (Jankowski, Collins and Campbell, 2008). To understand the entire phylogenetic relationship in the genus *Hydra*, we need a larger collection of hydra strains involving samples from Africa, Asia, Oceania, and South America.

4.2 Molecular phylogenetic relations between strains in a same group and a same sub-group in *vulgaris* group

In the *viridissima* group, North American strains (L9, M8) always made a monophyletic clade with the K10 strains originated in Switzerland as a sister group to the other all strains, M9, M10, and J8 in our analyses (Fig.1 – 3). M9 and M10 were collected in Israel and Danube, respectively. Although migration ability of hydra is low, hydra distributed in all continent without Antarctica. Therefore, it is reasonable to presume ancestor of hydra species originated before the separation of landmass, Pangea. Jankowski *et al*. (2008) suggested that the *viridissima* group and the *vulgaris* group were probably present before the separation of northern and southern landmasses. Before the North America and Europe were disrupted, they had made northern landmass, Laurasia. In the Laurasia, North America and West Europe were connected each other. If the ancestor of these *viridissima* group strains had originated in the middle part of the Laurasia (East Europe), it was probable that descendants dispersed to the west made the ancestors of L9, M8 strains and those dispersed to the east or stayed original location made the ancestors of M9, M10 strains. Recently, new green hydra, *H. sinensis*, was described from China (Wang *et. al.*, 2009). It is interesting to identify the phylogenetic position of this new species in the *viridissima* group.

In the *oligactis* group, Japanese strains (B1, B2, B3, L5, and P) formed a monophyletic clade against the other European (G7, K12) and North American (M4) strains (Fig. 1 and Supple. Fig. 1A - C). This result is consistent with Campbell's key (1983). According to Campbell's key, holotrichous isorhiza without transverse coils is also a trait of the *oligactis* group species excepting *Hydra pseudoligactis* (=*Hydra canadensis*). Such hydra possessing holotrichous isorhiza without transverse coils from Japan is *Hydra robusta* (= *Pelmatohydra robusta, Ito*), and from elsewhere is *Hydra oligactis*. As was the case of *viridissima* group, it was probable that ancestor of *oligactis* group strains originated in the middle part of Laurasia then dispersed to the west and east before the separation of the continent and diverged. *Hydra robusta* (B1, B2, B3, L5, and P) might be the descendant of such ancestor in the east end of the Laurasia, and the ancestor dispersed to the west might generate *Hydra oligactis* (G7, K12, M4).

In the *braueri* group, we only analyzed three species, *H. hymanae* (North America), *H. utahensis* (North America), and *H. circumcincta* (Europe). In our analyses, *circumcincta* (K8, M7) and *utahensis* (L10) made a monophyletic clade as a sister group to the *hymanae* (M1). We could not explain this inconsistency between the geographic distribution and phylogenetic relation among the *braueri* group species.

The genetic distance between strains within the *vulgaris* group was small (Fig. 1). However, three sub-groups were recognized distinctly in the *vulgaris* group (Fig. 1 - 3). The grouping of the three sub-groups, however, did not reflect the geographic distribution. Analyzed *vulgaris* sub-group strains all collected in Europe or Japan. In the *carne* sub-group strains, analyzed samples were collected from North America and Japan. *Hydra sp.* (K5, K6) sub-group strains were collected in Europe.

Of the *vulgaris* sub-group strains analyzed in this study, only four strains, B6, L2, K9, K7, were collected in Europe and all other strains (more than twenty) were collected in Japan. Although, three strains of them, B6, L2, and K9, formed a monophyletic clade, the strain K7 was clustered with other Japanese strains (Fig. 1 and Supple. Fig. 1A – C). Because only a small number of strains except for Japanese ones were analyzed in this study, we could not say much about the phylogenetic relationships in the *vulgaris* sub-group. However, it was suggested that members of the *vulgaris* sub-group were distributed in the Eurasia, but not in North America. The genetic distances between strains in the *vulgaris* sub-group were very small and the molecular

phylogenetic relations among *vulgaris* sub-group did not reflect the geographic distribution.

One of the other sub-groups consists of only two strains, K5 and K6, which had been identified as *Hydra attenuata* in the NIG collection. These strains had been cloned in 1962 and added to the NIG collection from Prof. Tardent's laboratory in 1988. However, Campbell (1989) suggested that *Hydra attenuata* was a synonym of *Hydra vulgaris*. Although K5 and K6 belong to *Hydra vulgaris* according to Campbell's suggestion, they had some morphological traits that were distinctly different from those of *Hydra vulgaris* as described in the results (Fig. 4 and 5). Therefore, we suggested that K5 and K6 form an independent sub-group in the *vulgaris* group.

All other strains in the *vulgaris* group belonged to the *carnea* sub-group in this molecular phylogenetic analysis. Although the M5 strain (USA CA) had been identified as *Hydra vulgaris* in the NIG collection, it belonged to the *carnea* sub-group by the molecular phylogenetic analyses. The M2 strain (USA CA), which had been identified as a member of the *vulgaris* group based on the morphological traits in this study, also belonged to the *carnea* sub-group. Although, only four stains, AEP, L4, M2, and M5, were analyzed in this study, the result suggested that there is only the *carnea* sub-group and no other sub-group of the *vulgaris* group in North America. There are, of course, several hydra species within the *vulgaris* group that have been described from North America, such as *H. littoralis, H. cauliculata, H. rutgersensis* (Campbell, 1983). It is interesting to see whether these North American *vulgaris* group species belong to the *carnea* sub-group or not. The M2 and M5 strains may be one of those species, because these two strains made a sister clade in relation to all other *carnea* sub-group (Fig. 1 and Supple. Fig 1A - C).

Although North American *vulgaris* group strains all belonged to the *carnea* sub-group, the distribution of the *carnea* sub-group was not restricted in the North America. Newly collected strains, FT 08, FO2 03, KAS 20, in Japan also belonged to the *carnea* sub-group.

4.3 Japanese *vulgaris* group hydra

Five species of hydra have been described from Japan (Ito, 1947a, b, c, d). Three of them, *H. magnipapillata*, *H. japonica*, and *H. paludicola*, belong to the *vulgaris* group, and maybe the same species. The standard wild type strain, *Hydra*

magnipapillata 105 (=B8 strain in this study), has been used in many researches. Recently, draft assemblies of the *Hydra magnipapillata* genome were generated (Chapman et al., 2010). Therefore, we thought it appropriate that there is only one hydra species belonging to the *vulgaris* sub-group in Japan, and it is *Hydra magnipapillata*.

However, in the course of our phylogenetic analyses, we found that newly collected hydra species from three different locations (Kasuga City, Tanushimaru in Kurume City and Ohori in Fukuoka City) in Fukuoka prefecture, Japan, belonged to the *carnea* sub-group (Fig. 1, FT 08, FO2 03, KAS 20). In the Japanese strains of the NIG collection, however, no strains belonged to the *carnea* sub-group, and all of them belonged to the *vulgaris* sub-group. We, of course, also collected *vulgaris* sub-group (*H. magnipapillata*) strains in Fukuoka, FO1 G, FO2 05, and ONI 5.Therefore, our molecular phylogenetic analyses suggested there were at least two species belonging to the *vulgaris* group in Japan, *Hydra magnipapillata* and *Hydra sp.* that belonged to the *carnea* sub-group. Members of the *vulgaris* sub-group are distributed in Eurasia but not in North America while those in the *carnea* sub-group are distributed in North America but not in Europe. Campbell (1983) described as well known *vulgaris* group species *Hydra littoralis* (North America), *Hydra attenuata* (= *Hydra vulgaris*) (Europe), and *Hydra magnipapillata* (Japan). Therefore, it is very interesting that both the *vulgaris* and the *carnea* sub-groups distribute in Japan.

However, most of the carnea sub-group samples in Japan were collected in urban area in the Fukuoka prefecture except the FT 08 strain. Therefore, we could not exclude the ability of anthropogenic dispersal from USA. However, we also could not exclude the ability a trans-Pacific distribution of the *carnea* sub-group hydra. We need information on hydra strains that inhabit Siberia and main land China for considering this interesting distribution of hydra species.

4.4 Hydra population in a pond

We found that, in all ponds from which multiple polyps were collected in our study, all polyps belonging to the *vulgaris* sub-group from the same pond had the same MtDNA nucleotide sequence. We cold not find any substitution in analyzed nucleotide sequences. The usual hydra reproduction system, asexual budding, provides a plausible explanation for this result. Interestingly, however, a *vulgaris* sub-group strain, FO2 05 (this sample represented three samples), and a *carnea* sub-group strain FO2 03 (this

sample also represented three samples) were collected in a same pond. The diameter of this small pond was about 20 m. We only analyzed the mitochondrial nucleotide sequence data. Therefore, we could not certain whether hybridization between FO2 05 (*vulgaris* sub-group) and FO2 03 (*carnea* sub-group) has occurred or not. To certain this problem is very intriguing.

4.5 Insertion in the mitochondrial DNA in *baurei* group

In the four species groups of hydra, insertions in mitochondrial DNA were observed only in the *braueri* group species. In the three other groups, *lrRNA, tRNA(W)* and *COII* coding region did not contain any insertions. This is a remarkable feature of the MtDNA evolution in the *braueri* group species.

In *H. utahensis* (L10) the inserted DNA suggested to have been originated from the *tRNA-COII* coding region based on the nucleotide sequence data (Supple. Fig. 4). The inserted location was between the *lrRNA* region and the *tRNA(W)* region. Duplication of this region and following modification was suggested. However, the inserted DNA sequence in *H. hymanae* (M1) did not any homology or similarity with any known DNA sequences. Therefore, it was suggested that origins of these insertions were different and the insertions had not occur in the common ancestor of the both strains. However, the MtDNA insertion of *H. hymanae* (M1) and of *H. utahensis* occurred in vicinity. The *lrRNA - tRNA(W) – COII* region in *braueri* group hydra may have a specific character of nucleotide sequence to induce insertions.

4.5 Substitution rate of nucleotide sequence is higher in mitochondrial genes than that of nuclear genes in hydra species

The difference of substitution rate between mitochondrial genome and nuclear genome has been an intriguing problem in the molecular evolutionary researches. The differences of substitution rate have been reported in several animals. Hydra polyps reproduce mainly asexually by budding, and have linear, not circular, mitochondrial genome. Therefore, it has been intriguing to investigate differences of the substitution rate between the mitochondrial genome and nuclear genome in hydra species.

Higher synonymous substitution rates in mitochondrial genes than in nuclear genes have been reported in animals. For example, it was reported that the rate of MtDNA evolution in primates is 5 to 10 times higher than in nuclear DNA (Brown et al.,

1979, 1982). Moriyama and Powell (1997) reported that mitochondrial genes have 1.7-3.4 times higher synonymous substitution rates than the fastest nuclear genes or 4.5-9.0 times higher rates than the average nuclear genes in *Drosophila.* On the other hand, lower Metazoa, such as Polrifera and Anthozoa, have been shown to have a reduced mitochondrial DNA substitution rate compared to nuclear DNA. In coral, 3 to 10 times slower rates of MtDNA sequence substitution compared with nuclear DNA were reported (reviewed by Shearer et al., 2002). Hellberg (2006) suggested that Anthozoa in Cnidaria showed slow synonymous substitution rate of MtDNA. However, Medusozoa, including Hydrozoa, showed faster rate as is the case of Bilateria.

Under a good condition with appropriate temperature and rich preys, hydra species reproduce rapidly by asexually budding. However, hydra species show sexual reproduction depending on the environmental condition. In the sexual reproduction phase, hydra species show diverse styles. Both monoecious species (the *viridissima* group, the *braueri* group, and some in the *vulgaris* group) and dioecious species (the *oligactis* group and most cases in the *vulgaris* group) with sexual differentiation have been reported. Because asexual reproduction is expected to decrease mutation rates at nuclear genes to retard accumulation of deleterious mutations (Kondrashov, 1988), it is interesting to know whether the ratio of synonymous rates in mitochondrial and nuclear genes differs from those in sexual species. Our result showed that this ratio in hydras was 2.4-7.8 and comparable to those in primates (Brown et al., 1979, 1982) and *Drosophila* (Moriyama and Powell, 1997). Therefore, partial asexual reproduction does not seem to reduce the nuclear mutation rate or some other factors such as lower mutation rates in MtDNA that have been observed in Anthozoa might have erased this effect.

In the estimation of time of speciation and phylogeographic events, knowing neutral evolutionary rates is a prerequisite. Furthermore, many questions in population genetics depend on information of mutation rates that can be estimated from neutral evolutionary rates (Kimura 1983). Finally, one of the main reasons why mitochondrial *CO1* was chosen for DNA barcoding in many animals is the higher evolutionary rate of mitochondrial genes in those species (Herbert et al., 2003). Therefore, it is important to obtain information of evolutionary rates when evolutionary questions are to be asked in hydras. Of course, we obtained only the ratio of the evolutionary rates in MtDNA and nuclear DNA, and not their absolute rates. This situation needs to be rectified in future

studies although we expect difficulties in calibrating time in organisms with soft bodies like hydras. Nevertheless, the higher rate in MtDNA compared to that in the nuclear genome in hydra as found in other animals indicate usefulness of MtDNA genes as markers for evolutionary studies of short time scales.

5. Conclusion

Species in the genus *Hydra* could be grouped into four monophyletic species groups by the molecular phylogenetic analyses in this study. The four hydra species groups were concordant with Campbell's suggested four groups, the *viridissima* group, *braueri* group, *oligactis* group and *vulgaris* group. Distinguishing between these four groups is easy by Campbell's keys based on the morphological traits. However, within each group it seems too difficult to distinguish between species only by the morphological key traits. As shown in this study, molecular data have an advantage in the identification of hydra species and phylogenetic analyses of the genus *Hydra*. However, we could only use several dozens samples from Europe, North America and Japan in this study, so in order to know the entire phylogenetic relationships in the genus *Hydra*, we need to conduct molecular phylogenetic studies on larger collections of hydra strains involving samples from Africa, Asia, Oceania, and South America.

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Figure captions

Figure 1:

Molecular phylogenetic tree (unrooted) based on maximum likelihood phylogenetic analysis of the combined mitochondrial dataset, containing the *COI* gene and *lrRNA-tRNA (W)-COII* genes. Numerals near the nodes indicate bootstrap values (%) based on 1000 replicates. Fifty-five strains of hydra were analyzed. The branch length indicator displays 0.05 substitutions per site. Four main clades, the *viridissima*, *braueri*, *oligactis* and *vulgaris* groups were recognized and indicated by the rectangular field with solid lines. Rectangular fields with dotted line indicate three sub-clades in the *vulgaris* group, *vulgaris* sub-group, *carnea* sub-group, and *Hydra sp.* (*K5, K6)* sub-group. These clades were also recognized by the NJ, MP, and BI analyses (See Supple. Fig. 1A, B, C). On the nodes of those clades, bootstrap values of the NJ (10000), MP (1000) analyses and posterior probabilities of the BI analysis are also indicated (%) with bold face (order; ML/NJ/MP/ BI).

Figure 2:

Molecular phylogenetic tree (unrooted) based on the maximum likelihood (ML) phylogenetic analysis of the *CnNOS1* gene. Numerals near the nodes indicate bootstrap values (%) based on 1000 replicates. Twenty-five strains of hydra were analyzed. The branch length indicator displays 0.02 substitutions per site. Four major clades were also recognized as in the case with the mitochondrial gene analyses (Fig. 1). On the nodes of major clades, which were recognized in NJ, MP, and BI analyses (See Supple. Fig. 2A, B, C), bootstrap values of the ML (1000), NJ (10000), MP (1000), analyses and posterior probabilities of the BI analysis (order; ML/NJ/MP/BI) were also indicated (%) with bold face.

Figure 3:

The molecular phylogenetic tree (unrooted) based on the maximum likelihood (ML) phylogenetic analysis of the *EF1a* gene. Twenty-five strains of hydra were analyzed. Numerals near the nodes indicate bootstrap values (%) based on 1000 replicates. The branch length indicator displays 0.01 substitutions per site. Four main clades were also recognized as in the case with the mitochondrial gene analyses (Fig.1).

On the nodes of major clades, which were recognized in NJ, MP, BI analyses (See Supple. Fig. 3A, B, C), bootstrap values of the ML (1000), NJ (10000), MP (1000), analyses and posterior probabilities of the BI analysis were indicated (%) with bold face (order; ML/NJ/MP/BI).

Figure 4:

A: Photograph of the strain K5. Length of tentacles is shorter than that of body column. Sexual polyps, male and female were also shown. B: Magnified testes. C: Magnified fertilized egg with embryonic theca.

Figure 5:

Four types of nematocysts in 8 strains of hydra species. Each strain shows distinctive shape of nematocysts. However, it is difficult to distinguish between *Hydra carnea* (L4) and *Hydra magnipapillata* (B8, 105).

Supplemental Figure 1:

A: Molecular phylogenetic tree based on the neighbor joining (NJ) phylogenetic analysis of the combined mitochondrial dataset, containing the *COI* gene locus and *lrRNA-tRNA (W)-COII* genes locus. The bootstrap consensus tree inferred from 10000 replicates is taken to represent the evolutionary history of the taxa analyzed. Numerals near the nodes indicate bootstrap values (%). Four main clades, the *viridissima, braueri, oligactis* and *vulgaris* groups were recognized and indicated by the rectangular field with solid lines. Rectangular fields with dotted line indicate three sub-clades in the *vulgaris* group, *vulgaris* sub-group, *carnea* sub-group, and *Hydra sp.* (*K5, K6)* sub-group. The ML, MP, and BI analyses also indicated these clades. On the nodes of those clades, bootstrap values of the NJ (10000), MP (1000), ML (1000) analyses and posterior probabilities of the BI analysis (order; NJ/MP/ML/BI) are indicated (%) with bold face. The branch length indicator displays 0.05 substitutions per site.

B: Maximum parsimony (MP) phylogenetic analysis of the combined mitochondrial dataset, containing *COI* gene and *lrRNA-tRNA (W)-COII* genes. Numerals near the nodes indicate bootstrap values (%) based on 1000 replicates. On the nodes of major clades, bootstrap values of the MP (1000), NJ (10000), ML (1000) analyses and posterior probabilities of the BI analysis (order; MP/NJ/ML/BI) are indicated (%) with bold face.

C: Bayesian inference (BI) analysis of the combined mitochondrial dataset, containing the *COI* gene and *COII* genes. Numerals near the nodes indicate posterior probabilities (%). On the nodes of major clades, posterior probabilities of the BI analysis and bootstrap values of the NJ (10000), MP (1000), ML (1000) analyses are indicated (%) with bold face (order; BI/NJ/MP/ML).

Supplemental Figure 2:

A: Molecular phylogenetic tree based on the neighbor joining (NJ) phylogenetic analysis of the *CnNOS1* gene. The bootstrap consensus tree inferred from 10000 replicates is shown. The evolutionary distances were computed using the Nei-Gojobori method and are in the units of the number of synonymous differences per synonymous site. Four major clades were also recognized as in the case with the mitochondrial gene analyses (Fig. 1). On the nodes of major clades, which were recognized in both MP, ML, and BI analyses, bootstrap values of the NJ (10000), MP (1000), ML (1000) analyses

and posterior probabilities of the BI analysis were indicated (%) with bold face (order; NJ/MP/ML/BI). The branch length indicator displays 0.05 substitutions per site.
B: Maximum parsimony (MP) phylogenetic analysis of the *CnNOS1* gene. Numerals near the nodes indicate bootstrap values (%). On the nodes of major clades, which were recognized in both NJ, ML, and BI analyses, bootstrap values of the MP (1000), NJ (10000), ML (1000) analyses and posterior probabilities of the BI analysis (%)were indicated with bold face (order; MP/NJ/ML/BI).
C: Bayesian inference (BI) analyses of the *CnNOS1* gene. Numerals near the nodes indicate posterior probabilities (%). On the nodes of major clades, which were recognized in NJ, MP, and ML analyses, posterior probabilities of the BI analysis and bootstrap values of the NJ (10000), MP (1000), ML (1000) analyses (%)were indicated with bold face (order; BI/NJ/MP/ML).

Supplemental Figure 3:
A: Molecular phylogenetic tree based on the neighbor joining (NJ) analysis of the *EF1a* gene. The bootstrap consensus tree inferred from 10000 replicates is shown. The evolutionary distances were computed using the Nei-Gojobori method and are in the units of the number of synonymous differences per synonymous site. Four main clades were also recognized as in the case with the mitochondrial gene analyses (Fig.1). On the nodes of major clades, which were recognized in both ML, MP, BI analyses, bootstrap values (%) of the NJ (10000), MP (1000), ML (1000) analyses and posterior probabilities of the BI analysis were indicated (%) with bold face (order; NJ/MP/ML/BI). The branch length indicator displays 0.05 substitutions per site.
B: Maximum parsimony (MP) phylogenetic analysis of the *EF1a* gene. Numerals near the nodes indicate bootstrap values (%). On the nodes of major clades, which were recognized in NJ, ML, BI analyses, bootstrap values (%) of the MP (1000), NJ (10000), ML (1000) analyses and posterior probabilities of the BI analysis were indicated (%) with bold face (order; MP/NJ/ML/BI).
C: Bayesian inference (BI) analysis of the *EF1a* gene. Numerals near the nodes indicate posterior probabilities (%). On the nodes of major clades, which were recognized in NJ, MP, ML analyses, posterior probabilities of the BI analysis and bootstrap values of the NJ (10000), MP (1000), ML (1000) were indicated (%) with bold face (order; BI/MP/NJ/ML).

Supplemental Figure 4:

Dot matrix between predicted amino acid sequence of an ORF (1200bp) in the *H. utahensis* (L10) MtDNA insertion and the amino acid sequence of the COII.

Tabale 1

Comparison between substitution rate of mitochondrial gene and of nuclear gene in *Hydra*

	viridissima group					*braueri* group	*oligactis* group			*vulgaris* group		
Compared strains pair	M9-L9	J8-L9	J8-K10	M9-K10	L9-K10	K8-L10	L5-B3	L5-G7	B3-G7	B8-J1	B8-L4	J1-L4
Synonimoous substitution rate of mitochondrial DNA (*C0I* and *COII*)	0.517	0.530	0.590	0.579	0.455	0.228	0.010	0.148	0.133	0.024	0.305	0.315
Synonimoous substitution rate of nuclear DNA (*CnNOS1* and *EF1a*)	0.208	0.208	0.184	0.184	0.102	0.069	0.040	0.020	0.017	0.010	0.051	0.051
Synonimoous substitution rate of mitochondrial DNA (*C0I* and *COII*) / Synonimoous substitution	2.486	2.543	3.207	3.147	4.461	3.304	4.000	7.400	7.824	2.400	5.000	6.176
											averge	4.329

CnNOS1 +Ef1a , Model : Codon: Modified Nei-Gojobori (Jukes-Cantor), Transition/Transversion Ratio : 2, Substitutions to Include : s: Synonymous only, No. of Sites : 367
COI +COII , Model : Codon: Modified Nei-Gojobori (Jukes-Cantor), Transition/Transversion Ratio : 2, Substitutions to Include : s: Synonymous only, No. of Sites : 353

Fig.1

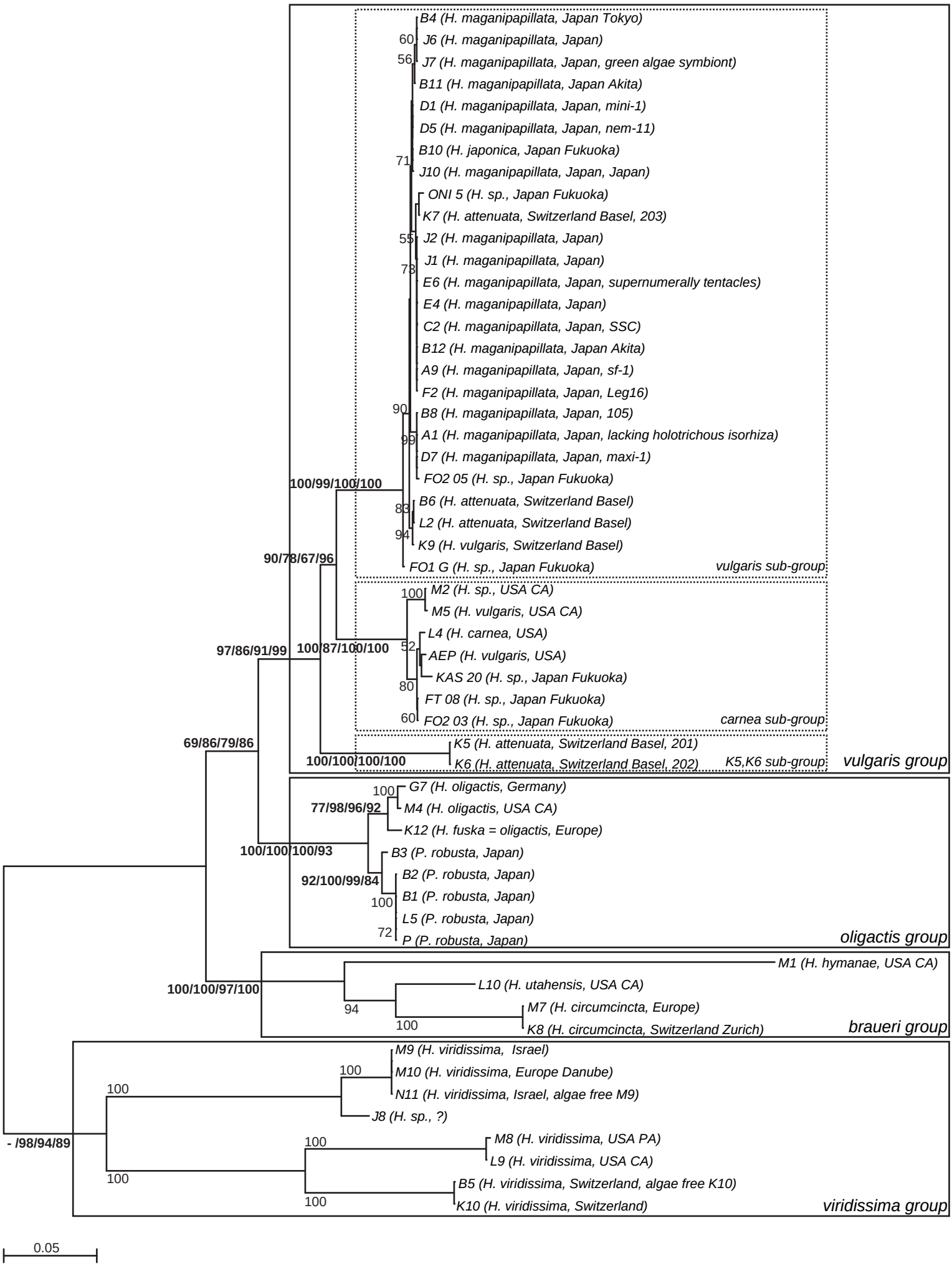

B4 (H. maganipapillata, Japan Tokyo)
J6 (H. maganipapillata, Japan)
J7 (H. maganipapillata, Japan, green algae symbiont)
B11 (H. maganipapillata, Japan Akita)
D1 (H. maganipapillata, Japan, mini-1)
D5 (H. maganipapillata, Japan, nem-11)
B10 (H. japonica, Japan Fukuoka)
J10 (H. maganipapillata, Japan, Japan)
ONI 5 (H. sp., Japan Fukuoka)
K7 (H. attenuata, Switzerland Basel, 203)
J2 (H. maganipapillata, Japan)
J1 (H. maganipapillata, Japan)
E6 (H. maganipapillata, Japan, supernumerally tentacles)
E4 (H. maganipapillata, Japan)
C2 (H. maganipapillata, Japan, SSC)
B12 (H. maganipapillata, Japan Akita)
A9 (H. maganipapillata, Japan, sf-1)
F2 (H. maganipapillata, Japan, Leg16)
B8 (H. maganipapillata, Japan, 105)
A1 (H. maganipapillata, Japan, lacking holotrichous isorhiza)
D7 (H. maganipapillata, Japan, maxi-1)
FO2 05 (H. sp., Japan Fukuoka)
B6 (H. attenuata, Switzerland Basel)
L2 (H. attenuata, Switzerland Basel)
K9 (H. vulgaris, Switzerland Basel)
FO1 G (H. sp., Japan Fukuoka)
vulgaris sub-group
M2 (H. sp., USA CA)
M5 (H. vulgaris, USA CA)
L4 (H. carnea, USA)
AEP (H. vulgaris, USA)
KAS 20 (H. sp., Japan Fukuoka)
FT 08 (H. sp., Japan Fukuoka)
FO2 03 (H. sp., Japan Fukuoka)
carnea sub-group
K5 (H. attenuata, Switzerland Basel, 201)
K6 (H. attenuata, Switzerland Basel, 202)
K5,K6 sub-group
vulgaris group
G7 (H. oligactis, Germany)
M4 (H. oligactis, USA CA)
K12 (H. fuska = oligactis, Europe)
B3 (P. robusta, Japan)
B2 (P. robusta, Japan)
B1 (P. robusta, Japan)
L5 (P. robusta, Japan)
P (P. robusta, Japan)
oligactis group
M1 (H. hymanae, USA CA)
L10 (H. utahensis, USA CA)
M7 (H. circumcincta, Europe)
K8 (H. circumcincta, Switzerland Zurich)
braueri group
M9 (H. viridissima, Israel)
M10 (H. viridissima, Europe Danube)
N11 (H. viridissima, Israel, algae free M9)
J8 (H. sp., ?)
M8 (H. viridissima, USA PA)
L9 (H. viridissima, USA CA)
B5 (H. viridissima, Switzerland, algae free K10)
K10 (H. viridissima, Switzerland)
viridissima group
100/99/100/100
90/78/67/96
97/86/91/99
100/87/100/100
69/86/79/86
100/100/100/100
100/100/100/93
77/98/96/92
92/100/99/84
100/100/97/100
- /98/94/89
0.05

Fig.2

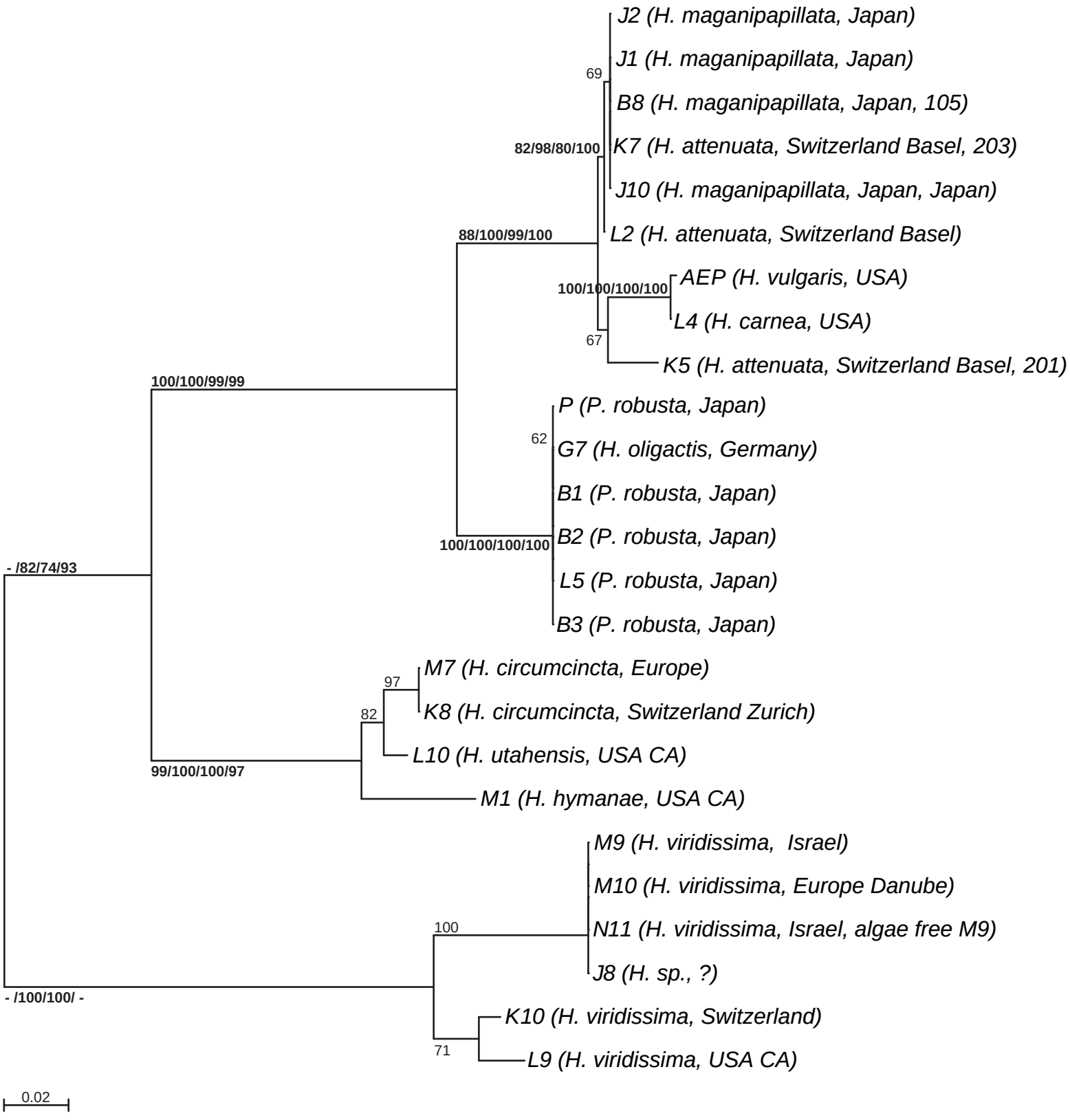

J2 (H. maganipapillata, Japan)
J1 (H. maganipapillata, Japan)
69
B8 (H. maganipapillata, Japan, 105)
82/98/80/100
K7 (H. attenuata, Switzerland Basel, 203)
J10 (H. maganipapillata, Japan, Japan)
L2 (H. attenuata, Switzerland Basel)
88/100/99/100
AEP (H. vulgaris, USA)
100/100/100/100
L4 (H. carnea, USA)
67
K5 (H. attenuata, Switzerland Basel, 201)
100/100/99/99
P (P. robusta, Japan)
62
G7 (H. oligactis, Germany)
B1 (P. robusta, Japan)
B2 (P. robusta, Japan)
100/100/100/100
- /82/74/93
L5 (P. robusta, Japan)
B3 (P. robusta, Japan)
M7 (H. circumcincta, Europe)
97
K8 (H. circumcincta, Switzerland Zurich)
82
L10 (H. utahensis, USA CA)
99/100/100/97
M1 (H. hymanae, USA CA)
M9 (H. viridissima, Israel)
M10 (H. viridissima, Europe Danube)
100
N11 (H. viridissima, Israel, algae free M9)
J8 (H. sp., ?)
- /100/100/ -
K10 (H. viridissima, Switzerland)
71
L9 (H. viridissima, USA CA)
0.02

Fig.3

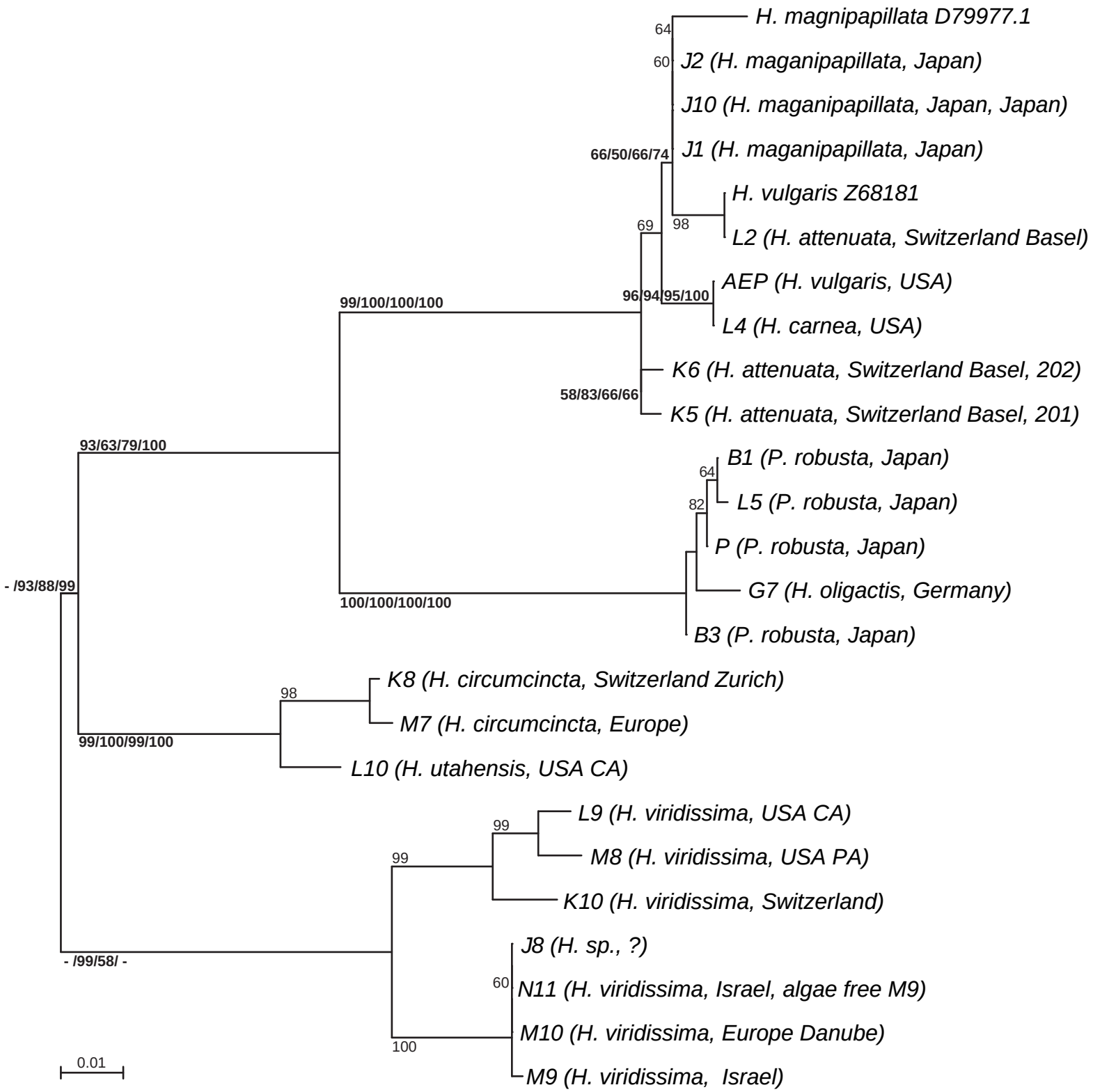

H. magnipapillata D79977.1
64
60
J2 (H. maganipapillata, Japan)
J10 (H. maganipapillata, Japan, Japan)
66/50/66/74
J1 (H. maganipapillata, Japan)
H. vulgaris Z68181
69
98
L2 (H. attenuata, Switzerland Basel)
AEP (H. vulgaris, USA)
96/94/95/100
99/100/100/100
L4 (H. carnea, USA)
K6 (H. attenuata, Switzerland Basel, 202)
58/83/66/66
K5 (H. attenuata, Switzerland Basel, 201)
93/63/79/100
B1 (P. robusta, Japan)
64
L5 (P. robusta, Japan)
82
P (P. robusta, Japan)
- /93/88/99
G7 (H. oligactis, Germany)
100/100/100/100
B3 (P. robusta, Japan)
K8 (H. circumcincta, Switzerland Zurich)
98
M7 (H. circumcincta, Europe)
99/100/99/100
L10 (H. utahensis, USA CA)
L9 (H. viridissima, USA CA)
99
M8 (H. viridissima, USA PA)
99
K10 (H. viridissima, Switzerland)
J8 (H. sp., ?)
- /99/58/ -
60
N11 (H. viridissima, Israel, algae free M9)
M10 (H. viridissima, Europe Danube)
100
M9 (H. viridissima, Israel)
0.01

Fig.4

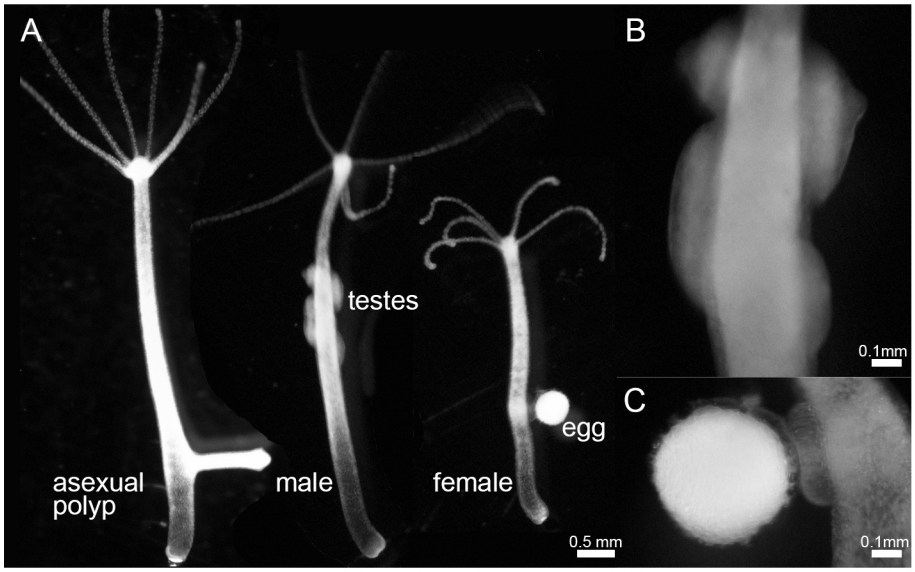
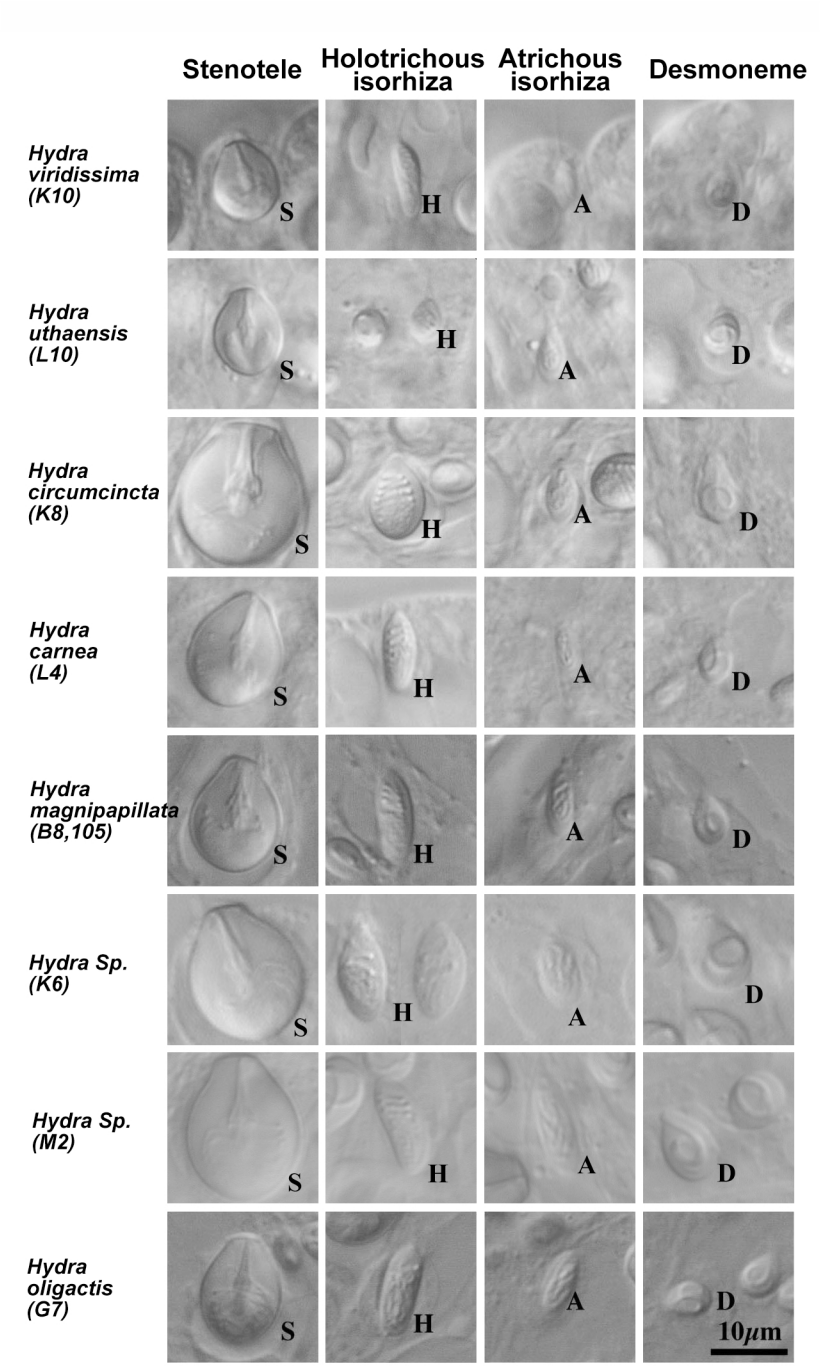
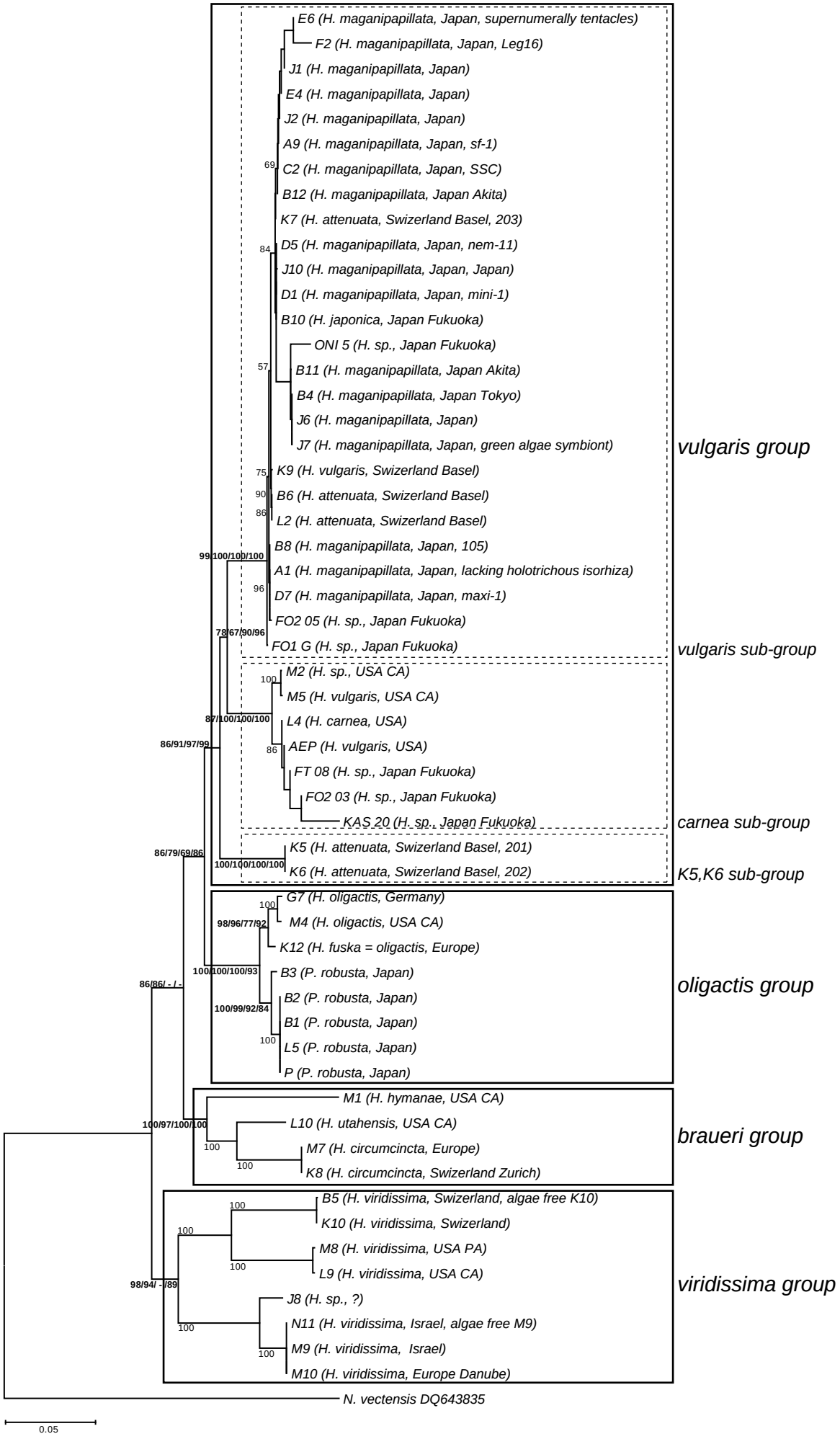


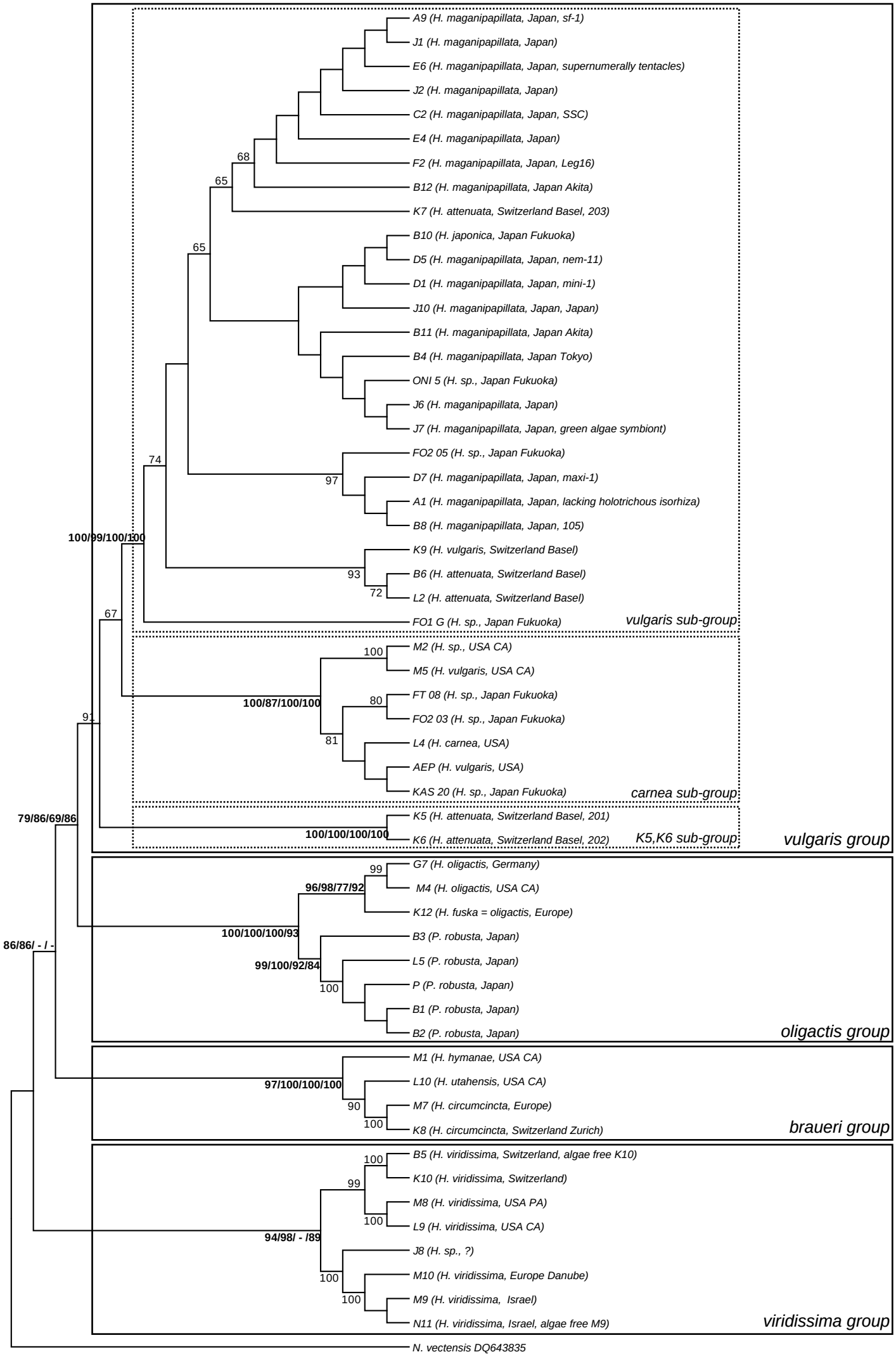
Fig.5


Stenotele
Holotrichous isorhiza
Atrichous isorhiza
Desmoneme
Hydra viridissima (K10)
Hydra uthaensis (L10)
Hydra circumcincta (K8)
Hydra carnea (L4)
Hydra magnipapillata (B8,105)
Hydra Sp. (K6)
Hydra Sp. (M2)
Hydra oligactis (G7)
S
H
A
D
10μm

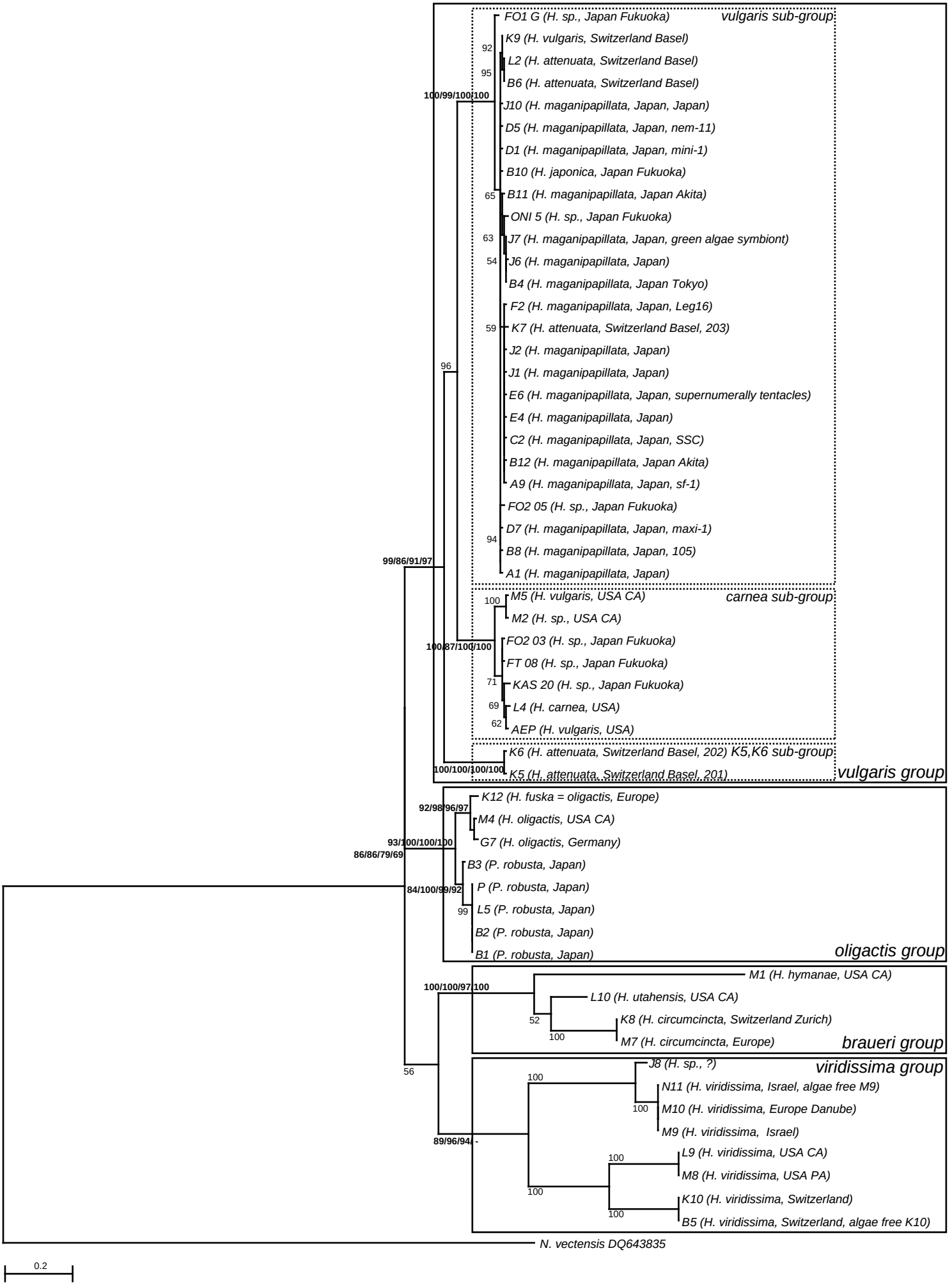
Supplemental Fig.1A



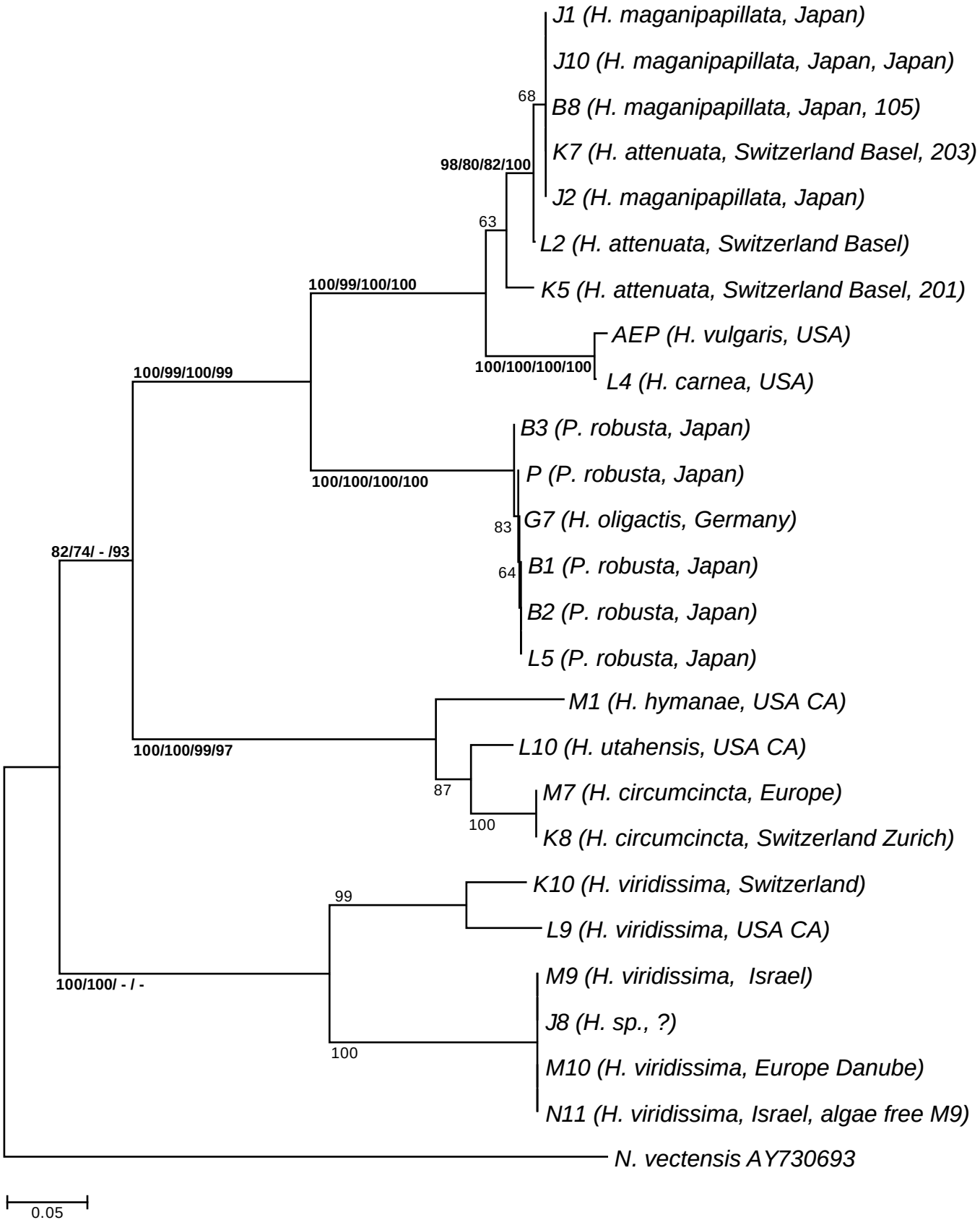
Supplemental Fig.1B



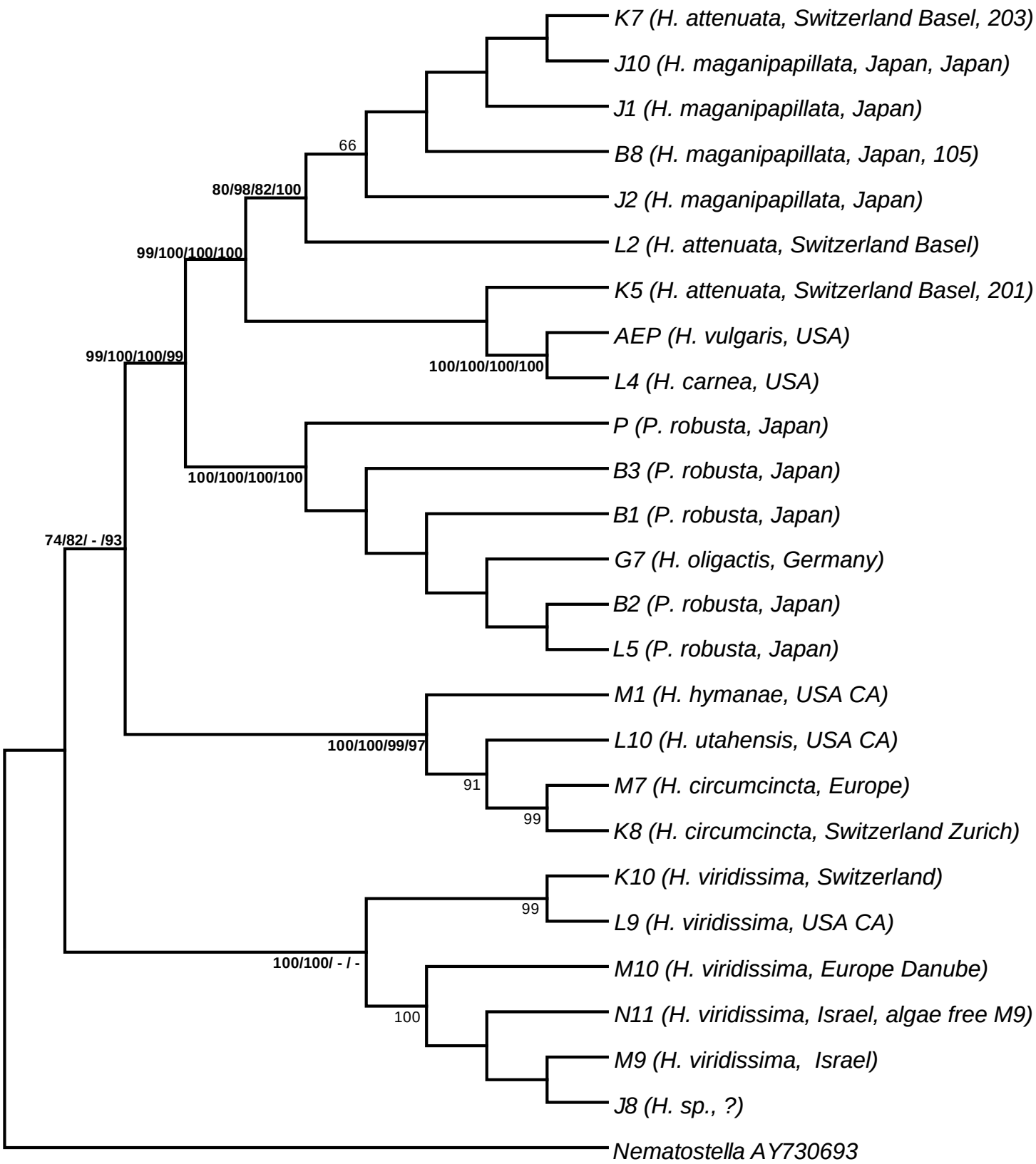
Supplemental Fig.1C



Supplemental Fig.2A

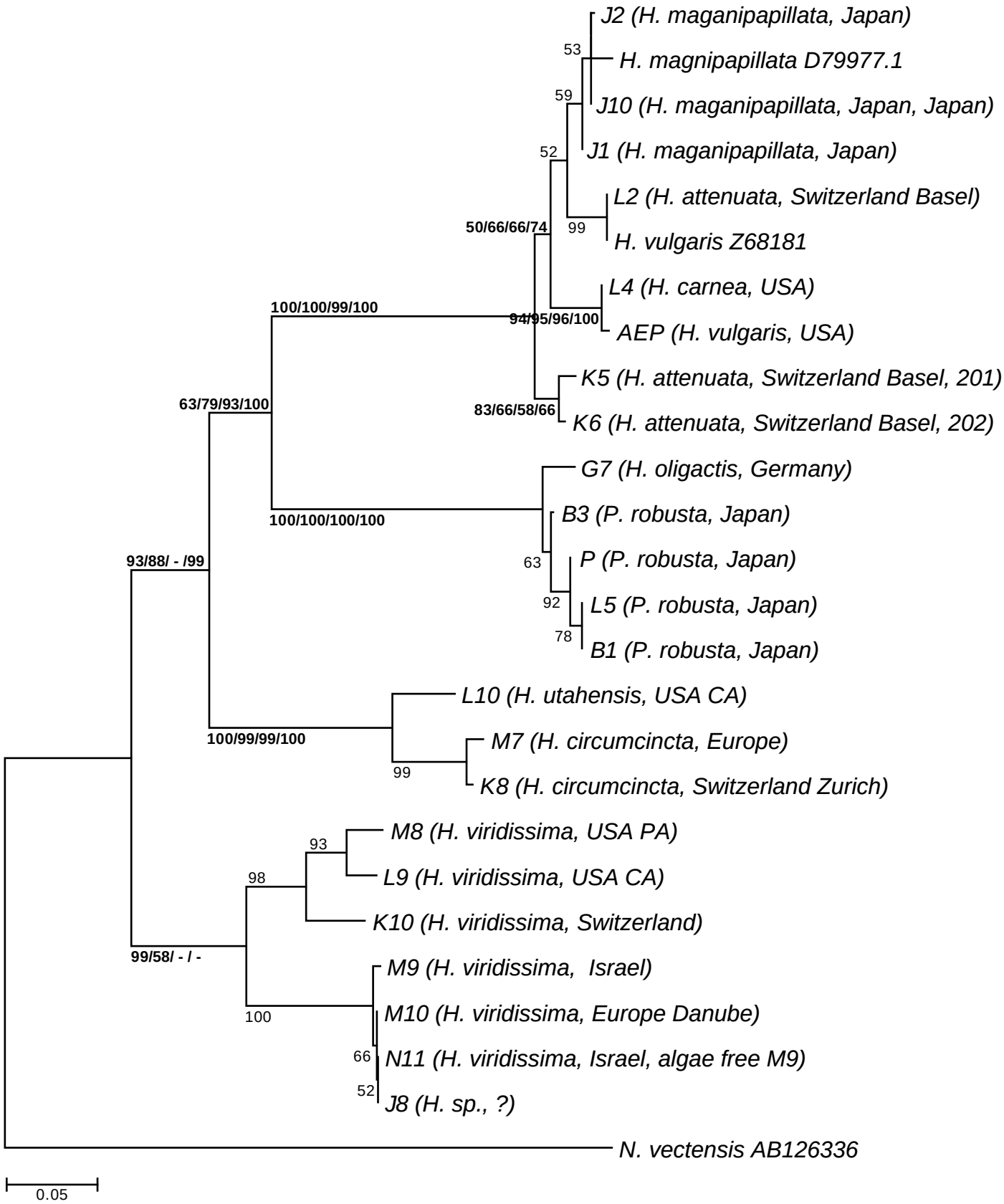


Supplemental Fig.2B

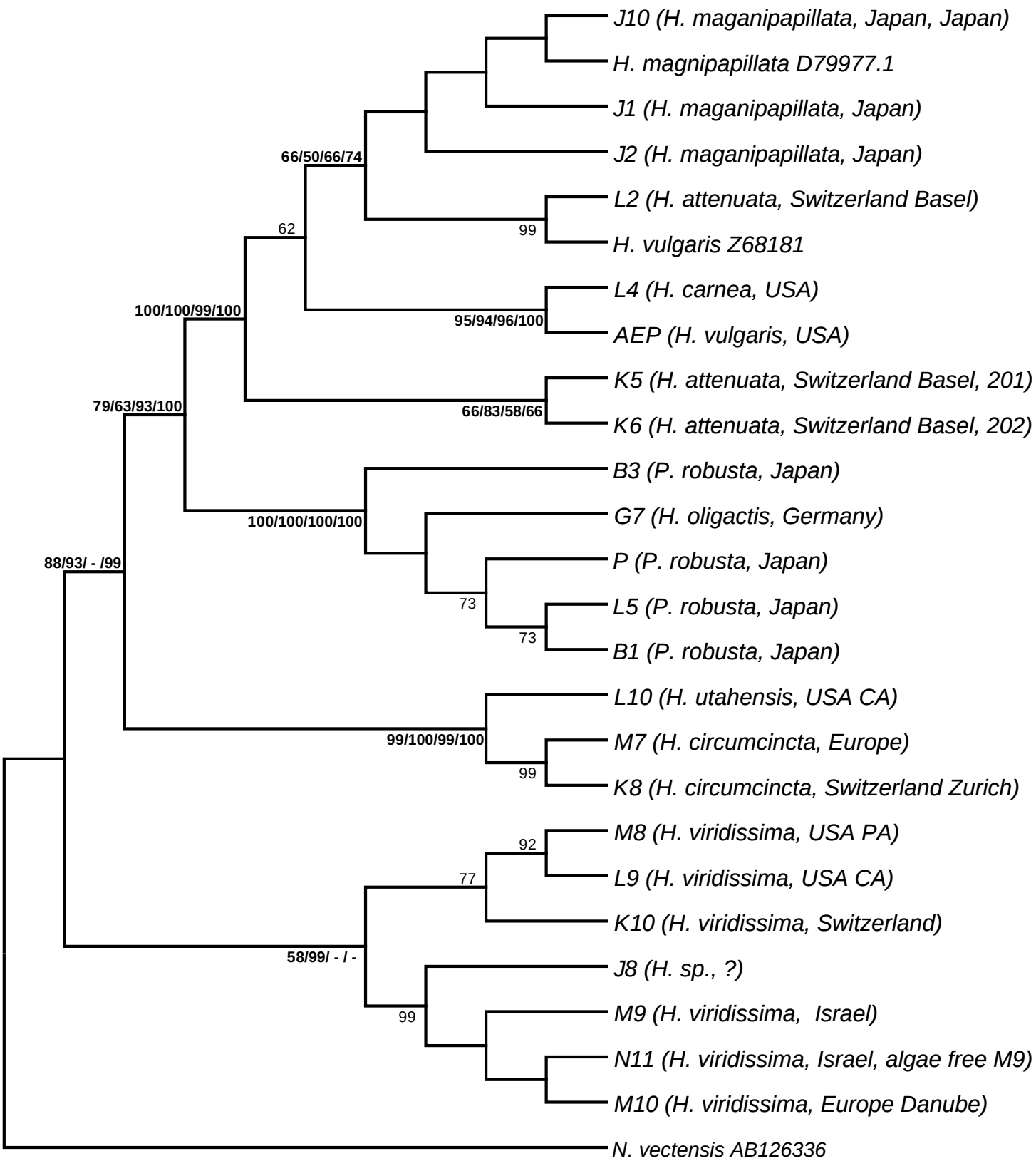


Supplemental Fig.2C

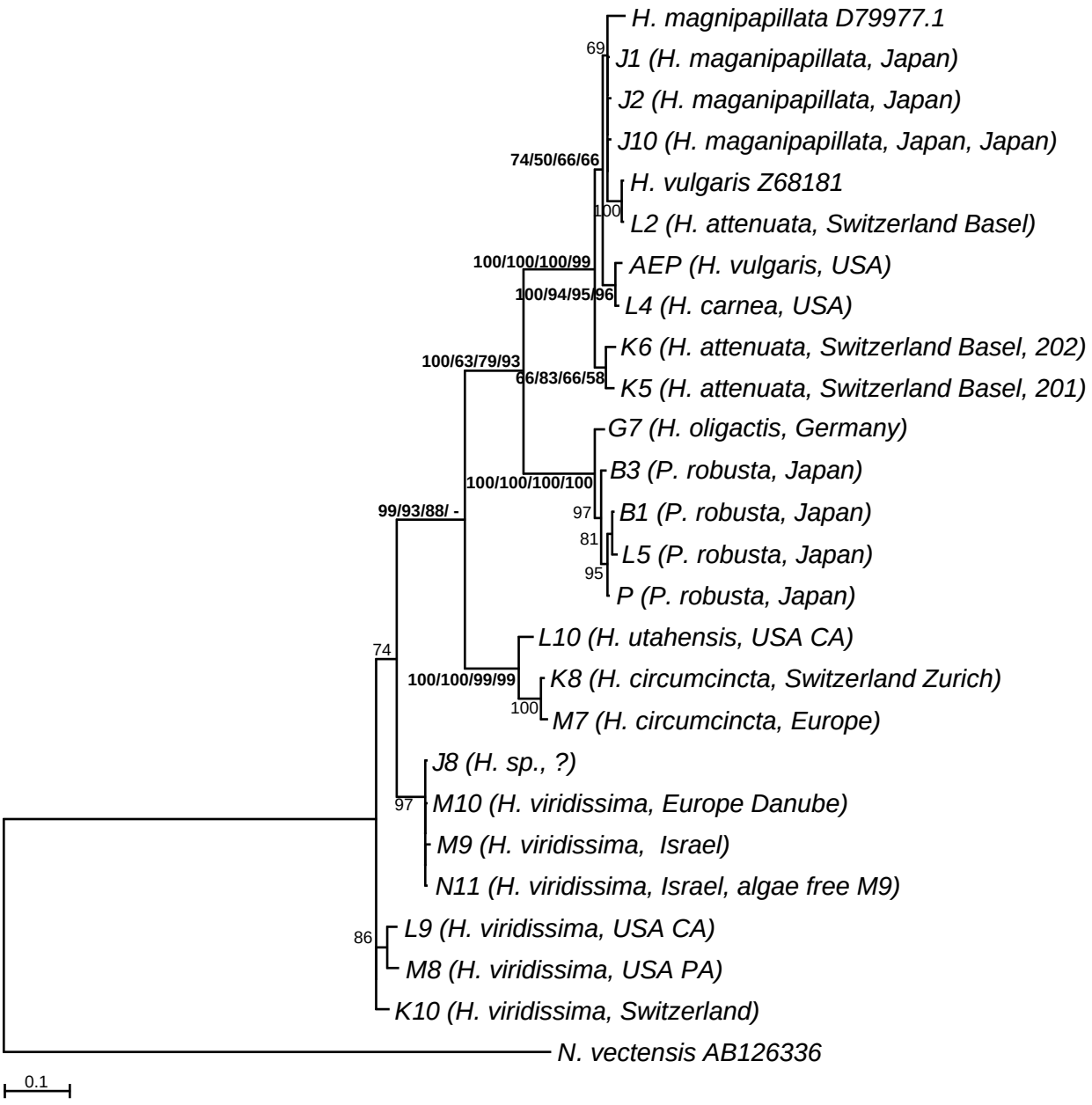
Supplemental Fig.3A



Supplemental Fig.3B



Supplemental Fig.3C



Supplemental Fig.4